2019

Microbial Communities Associated with Dragonfly Nymphs Raised in Varying Concentrations of Amoxicillin

Michelle Basha
University of Mississippi

MICROBIAL COMMUNITIES ASSOCIATED WITH DRAGONFLY NYMPHS RAISED IN VARYING CONCENTRATIONS OF AMOXICILLIN

By

Michelle Basha

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford May 2019

Approved by

_____

Advisor: Dr. Colin Jackson

_____

Reader: Dr. Peter Zee

_____

Reader: Dr. Erik Hom

ACKNOWLEDGEMENTS

I would like to extend my most sincere thanks to Dr. Colin Jackson for guiding me through this process and showing me an immense amount of patience throughout the research and writing of this thesis. Your mentorship and willingness to share your expertise has greatly impacted my time as an undergraduate. I could not have asked for a better advisor. I would like to thank the University of Mississippi Biology Department for equipping me with the resources to further my education in my pursuit of a career as a physician. I would like to thank the Sally McDonnell Barksdale Honors College for their support in the funding of this research and for the many other educational opportunities they've presented me with over the past four years. I'd like to thank Sarah Nobles for all of her help in both the lab and the field and for teaching me the importance of vigilance and precision in research. Our many hours of catching dragonflies will not be forgotten. I'd also like to thank Eric Weingarten for all of his help and patience and in always challenging me to dig deeper in my understanding of science. I'd also like to thank my additional readers, Dr. Peter Zee and Dr. Erik Hom, for assisting me with my thesis. I sincerely thank my parents for raising me to be inquisitive and hardworking and for their immeasurable support during my time at the University of Mississippi.

ABSTRACT

Microbial Communities Associated with Dragonfly Nymphs Raised in Varying Concentrations of Amoxicillin

The bacteria on and within an organism make up that organism's microbiome. Given interest in the use of antibiotics in agriculture and the effect of microbes on human health, more studies are needed on the microbial community composition of different organisms and how it responds to antibiotic use. This study investigated changes in the amount of antibiotic resistant bacteria present in dragonfly nymphs exposed to differing concentrations of amoxicillin. Next generation sequencing of the 16S rRNA gene was used to identify cultures of these antibiotic resistant bacteria. Increasing the concentration of antibiotics the dragonfly nymphs were exposed to resulted in greater numbers of antibiotic resistant bacteria. From both amoxicillin + TSA and TSA-only plates, Proteobacteria was the most abundant phyla detected. Bacteroidetes was the major phyla detected in nymphs raised in 0% amoxicillin and plated on amoxicillin + TSA plates. There was a high relative proportion of members of the phylum Firmicutes in all samples plated on TSA plates. In the nymphs raised in 0% amoxicillin plated on TSA plates, members of Firmicutes made up the majority of their microbiome. This study demonstrates that the bacterial communities associated with dragonfly nymphs are affected by changes in the environment, and that exposure to antibiotic pollution likely increases the number of antibiotic resistant bacteria within aquatic insects.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

Introduction

Despite being discovered by van Leeuwenhoek in the 17th century, humankind's understanding of the microbial world is still largely limited (Pace 1997). Carl Woese's phylogenetic work organizing organisms into three divisions (Eukarya, Bacteria, Archaea) based on 16S ribosomal RNA (rRNA) revolutionized the understanding of microbial diversity (Woese, 1987). Following Woese's work, Norman Pace used 16S rRNA approaches on samples collected directly from the environment, eliminating the need for culture-based methods, and expanding our understanding of microbial diversity (Pace, 1997). Over the past decade alone, the development of high-throughput sequencing technologies has resulted in a mass increase of our appreciation of microbial diversity (Waldor et al., 2015).

Over the same time period that there has been an increased understanding of microbial diversity, there has been an increased use of antibiotics. In the United States in 2010, there was an estimated antibiotic prescription rate of 506 per 1000 individuals in the population annually, of which only 353 were likely appropriately prescribed (Fleming-Dutra et al., 2016). This increased rate of prescribing antibiotics has led to antibiotic resistant bacteria (ARB) becoming of increasing concern (CDC, 2014). Resistance to antibiotics has been developing since Alexander Fleming discovered penicillin in 1928 (Fleming, 1944). While the incidence of antibiotic-resistant infections continues to rise in the United States, there has been a decline in research and in the development of new antibiotics in response to this epidemic (Spellberg et al., 2008). Antibiotic resistance of clinically significant bacterial pathogens based on data from hospitals across the US was found to be highest in/for *Enterococcus faecium*/vancomycin (87.1%), *Staphylococcus aureus*/oxacillin–methicillin (56.8%), and *S.*

aureus/clindamycin (39.7%), which are also the most common pathogen/antibiotic pairs (Edelsberg et al., 2014). Antibiotics are not just limited to clinical settings and can also show up in the environment. For example, trace amounts of antibiotics have been detected from wastewater treatment plants (Liu et al., 2014), and these treatment plants may contain ARB.

Antibiotic use in agriculture has also contributed to antibiotic resistance being a public health concern in humans (Chang et al., 2015). The main use of antibiotics in the agricultural industry is giving livestock many antibiotics to promote growth and to prevent infection (Khachatourians, 1998). Potential mechanisms of passing antibiotic resistance from agriculturally associated bacteria to those associated with humans include direct infection from an animal source, or the transfer of antibiotic resistant genes to human pathogens (Chang et al., 2015). For some years, antibiotic use in agriculture has exceeded that in medicine (FDA, 2012) and these antibiotics can enter agricultural runoff. Agricultural runoff can enter local water systems (Wang et al., 2016), which can affect microorganisms in these systems through the horizontal transfer of genetic material, including antibiotic resistance genes (Davies, 1994). As a result of horizontal gene transfer, ARB have been found in water and sediment as well as in aquatic organisms (Cabello, 2006). These organisms are involved in different parts of aquatic food chains, providing a further mechanism for conferring antibiotic resistant bacteria to other organisms.

As with other organisms, communities of bacteria are naturally associated with dragonflies and constitute their microbiome. The hemimetamorphic life cycle of dragonflies includes both an aquatic nymph stage and a terrestrial adult stage (Glaser,

2007; May, 2013). The nymphs' exposure to antibiotics in aquatic systems could result in them obtaining antibiotic resistant bacteria from their environment (Uilenburg et al. 2006). Dragonflies play a role in food webs, as they are preyed upon by fish, birds and other dragonflies (Knight et al., 2005), and these organisms are further preyed upon by other organisms. If dragonflies do indeed host ARB, this could serve as another method of transmission of ARB, especially if these bacteria are retained after metamorphosis into the adult. These antibiotic resistant bacteria could then be transmitted over long distances, as some adult dragonflies can migrate over hundreds to thousands of kilometers (May, 2013). One question, though, is whether dragonflies can even harbor ARB and if exposure to antibiotics selects for greater numbers of ARB in these organisms. This question led to the development of this study, which aimed to determine and compare the bacterial communities and their antibiotic resistance in dragonfly nymphs exposed to different concentrations of the antibiotic amoxicillin.

Methods

Sample Collection

25 dragonfly nymphs, approximately 5 cm long and of similar appearance, were obtained from pond 7 at the University of Mississippi Field Station in Abbeville, Mississippi on July 19, 2017 from 1:30-2:30 pm. The weather was 33° C and sunny, and the pond water level was approximately 29 cm. Nymphs were collected by dip-netting, removed from foliage with tweezers and placed in sterile collecting jars along with a small volume of pond water. The samples were then transported back to the laboratory at the University of Mississippi main campus in Oxford, Mississippi.

Experimental Exposure to Antibiotic Treatment

Dragonfly nymphs were raised individually in microcosms with differing concentrations of amoxicillin. Microcosms consisted of sterilized (70% ethanol) plastic containers with a maximum volume of approximately 2 L with mesh netting placed on top. Approximately 325 mL of sterilized gravel was placed into each microcosm. Amoxicillin was dissolved in pond water and poured into the plastic containers through a strainer to remove suspended particles. Each microcosm was filled with 500 mL of RO water and 500 mL of pond water. The minimum inhibitory concentration (MIC) of amoxicillin was determined to be 0.016 g by methods outlined by Andrews (2001). Thus, five nymphs were raised in water with no amoxicillin (control), five at the MIC, five at 2xMIC, and five at 5xMIC (i.e. four concentrations, with five replicates at each concentration). Experimental treatments ran for 10 d, and on each day nymph activity level was monitored and recorded, as was any nymph death. The dragonflies were not fed

during the duration of this experiment. After 10 d exposure to the experimental treatments, any living nymphs were processed for dissection.

Nymph Processing

Surviving nymphs were removed from containers and processed. Nymphs were dissected by slicing each surviving nymph sagittally with a straight razor using sterile techniques. Each whole nymph was placed in a centrifuge tube containing 1 ml of 0.85% sterile saline solution and vortexed for five minutes. A subsample of the liquid from the suspension was removed for plate counts of antibiotic resistant bacteria. This subsample was serially diluted in sterile saline and spread plated onto tryptic soy agar (TSA) plates containing no antibiotics and onto TSA plates amended with 3x MIC of amoxicillin. Plates were incubated at 37°C for 48 h. After 48 h, the number of visible colonies on each plate were counted. For each sample, colonies on the plate showing the greatest growth were scraped/washed with sterile saline into a 1.5 ml tube for determination of colony identity through 16S rRNA gene sequencing. Tubes were centrifuged (8,000 xg, 10 min), the supernatant removed, and the pellets frozen prior to DNA extraction.

DNA Extraction and 16S rRNA Gene Sequencing

The frozen samples for microbiome analysis were thawed, and DNA was extracted using a PowerSoil DNA Isolation Kit (Mo Bio Laboratories Inc. Carlsbad, CA). Agarose gel electrophoresis was used to confirm the presence of recovered DNA. A dual-index barcoded Illumina next-generation sequencing approach was used to amplify and sequence the V4 region of the 16S rRNA gene (Kozich et al., 2013, Jackson et al., 2015)

which was sequenced at the University of Mississippi Medical Center (UMMC) Molecular and Genomics Core Facility on an Illumina MiSeq platform. Samples ma01-ma16 were obtained from antibiotic plates, while samples mt01-mt16 were from TSA plates.

Bioinformatics Sequence Analysis

The bioinformatics software mothur (Schloss et al. 2009) was used to analyze 16S rRNA sequence data. A series of system commands were used to remove sequences errors and align sequences against a 16S rRNA sequence database. VSEARCH software was used to remove chimeras, sequences which incorrectly combine during PCR amplification. The RDP sequence database was used to classify the remaining aligned sequences and contaminant sequences (those identified as Eukarya, Archaea, chloroplast, mitochondria, or unknown) were removed from the dataset. Bacterial sequences were then grouped into closely related individuals, operational taxonomic units (OTUs), which were determined by 97% sequence similarity (Schloss et al., 2009). Analysis of each bacterial community from each sample was performed based on the presence and relative abundance of these OTUs. Analysis of Variance (ANOVA) was used to examine differences in the composition of bacterial taxa in the nymphs at differing concentrations of amoxicillin and to compare ARB at each concentration.

Results

All nymphs yield colonies of bacteria on both TSA and TSA + amoxicillin plates (Table 1). Nymphs raised in the same concentration of antibiotic had similar numbers of colony forming units (CFUs) on both TSA and TSA + amoxicillin plates. On the amoxicillin plates, the nymph with the least number of amoxicillin resistant bacteria was raised in 0% amoxicillin (N4), yielding $1.5x10^3$ CFUs per nymph. The nymph with the most amoxicillin resistant bacteria was raised in 5xMIC amoxicillin (N16), yielding $2.64x10^7$ CFUs. On the TSA-only plates, the sample from the nymph that displayed the most growth was raised in 5xMIC amoxicillin (N16), yielding $2.33x10^7$ CFUs. The sample plated on a TSA plate displaying the least amount of growth was raised in the MIC of amoxicillin (N7), yielding $9x10^3$ CFUs.

The mean number of CFUs on both TSA + amoxicillin and TSA only plates increased with increasing dosage of amoxicillin to which the nymphs were exposed (Figure 1). This effect of antibiotic concentration was significant for both amoxicillin + TSA plates (ANOVA, $p=0.05$) and TSA plates (ANOVA, $p=0.001$).

The most common bacterial phylum identified in cultures from amoxicillin plates, was Proteobacteria (Table 2). Other major phyla and subphyla included Firmicutes and Bacteroidetes (Table 2). While Proteobacteria was the most abundant phylum in each sample set, the portions of other major phyla varied from sample to sample, even within replicates of the same treatment type. For example, Bacteroidetes made up 23.4% of the sequences obtained from the ma03 sample, but only 3.9% of sequences from the ma02 sample.

Nymph	Concentration of Amoxicillin	Growth on Amoxicillin (CFUs)	Growth on TSA (CFUs)
N1	0	9.00E+03	3.10E+04
N2	0	1.10E+04	1.40E+06
N3	0	4.00E+03	1.00E+06
N4	0	1.59E+03	5.40E+04
N5	MIC	7.40E+06	1.46E+07
N6	MIC	1.95E+05	1.70E+06
N7	MIC	4.40E+04	9.00E+03
N8	MIC	1.20E+06	1.20E+06
N9	2xMIC	3.50E+06	3.50E+06
N10	2xMIC	4.00E+05	2.00E+06
N11	2xMIC	1.00E+06	2.10E+06
N12	2xMIC	5.00E+06	6.90E+06
N13	5xMIC	7.20E+06	1.25E+07
N14	5xMIC	1.15E+07	1.33E+07
N15	5xMIC	1.90E+06	1.46E+07
N16	5xMIC	2.64E+07	2.33E+07

Table 1. Numbers of colony forming units (CFUs) obtained from dragonfly nymphs (N1-N16) raised in different concentrations of amoxicillin for 10 d. A 0 indicates no amoxicillin, MIC is the minimum inhibitory concentration of amoxicillin, and 2x MIC and 5x MIC, represent twice and five times the MIC, respectively. Growth on Amoxicillin is the number of CFUs obtained on TSA plates amended with amoxicillin while Growth on TSA is the number of CFUs obtained on regular TSA plates.

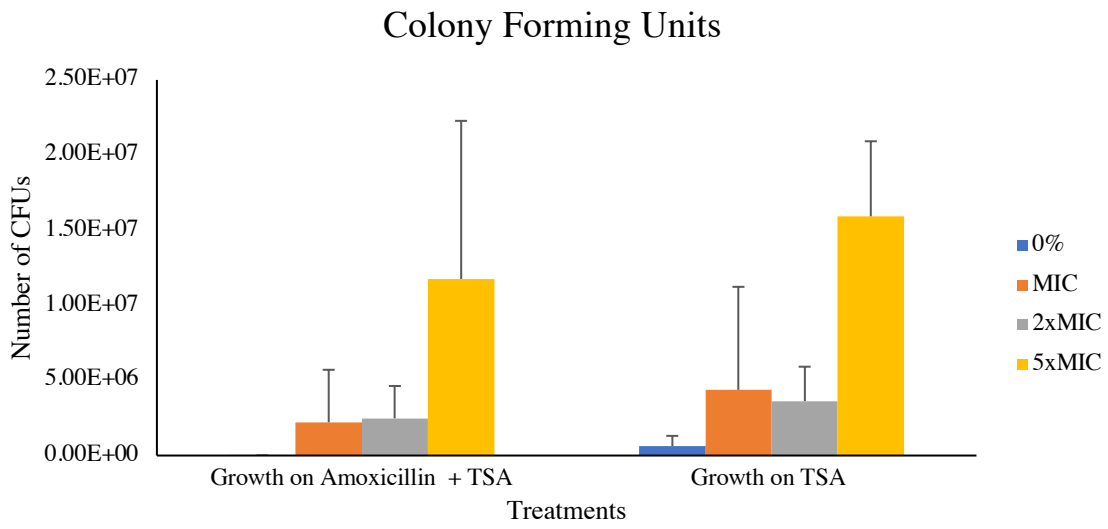

Figure 1. Mean number of CFUs obtained from dragonfly nymphs exposed for 10 days to different doses of amoxicillin (0, MIC, 2xMIC, 5xMIC). Values are means (plus standard deviation) for four nymphs in each treatment and are shown for growth on amoxicillin + TSA plates (left) compared to grown on TSA only plates (right). The error bars represent the spread of the data within each treatment group around the mean value.

Taxon	0%	MIC	2xMIC	5xMIC
Acidobacteria	0.0075	0.0025	0.0025	0
Actinobacteria	0.0125	0	0.0075	0
Armatimonadetes	0	0	0.0025	0
Bacteria_unclassified	0.16	0.005	0.035	0.005
Bacteroidetes	7.2475	0.0975	0.035	0.0175
Chloroflexi	0	0.0025	0	0
Deinococcus-Thermus	0	0	0.0025	0
Firmicutes	0.035	0.0775	0.3175	0.045
Planctomycetes	0.0975	0	0.0025	0
Proteobacteria	92.435	99.805	99.58	99.9325
Verrucomicrobia	0.0075	0.0025	0.0225	0

Table 2. Percentage of major bacterial phyla and subphyla in mixed cultures obtained from dragonfly nymphs raised on amoxicillin plates. The averages of the four surviving nymphs in each of the four treatment groups (0% amoxicillin, MIC, 2xMIC, 5xMIC amoxicillin) are shown.

Proteobacteria was also the most common phylum detected in cultures on TSA plates (Table 3). In these samples, the proportions of dominant phyla in each sample were more similar based on the concentration of antibiotic that nymph was raised in. For example, mt01-04 were raised without antibiotics and grown on TSA plates. Their relative proportion of Firmicutes were 63.1%, 59.6%, 81.1%, and 65.8%. mt09-12 were raised in 2xMIC amoxicillin, and their relative proportions of Firmicutes were 6.9%, 22.3%, 10.8%, and 22.1%. The proportion of Firmicutes decreased and the proportion of Proteobacteria increased as the concentration of amoxicillin increased above the MIC in samples from TSA plates (Table 3). However, the proportion of Bacteroidetes is minimal to none in samples from nymphs raised in any concentration of amoxicillin (MIC-5xMIC), whereas it varies in nymphs raised in no amoxicillin.

Operational taxonomic units (OTUs) were classified based on 97% 16S rRNA similarity. The number of OTUs found in each sample varied (Figure 2), ranging from 19 OTUs in cultures derived from a TSA plate from a nymph raised without amoxicillin to three OTUs on a TSA + amoxicillin plate from a nymph raised in 5xMIC amoxicillin. There were differences in the numbers of OTUs across the MIC levels for both treatments. Counts of species on TSA plates were much higher than on TSA + amoxicillin plates. For example, in the treatment group raised in 0% amoxicillin, counts on TSA plates were nearly five times as high as counts on amoxicillin + TSA plates. There is also a general trend of decreased number of OTUs/richness with increased antibiotic exposure. For the amoxicillin + TSA-plated samples, the number of OTUs in the 0% amoxicillin treatment group is roughly 10.5, whereas it is only 4 in the 5xMIC treatment group. The same trend can be seen in TSA-only plated groups. The number of

OTUs in the 0% amoxicillin treatment group is roughly 60, whereas it is half that in the 5xMIC treatment group.

Non-metric multidimensional scaling (NMDS) (Figure 3) was used to spatially compare the similarities of the bacterial cultures obtained from dragonfly nymphs raised in each of the treatment groups. Samples primarily separate by the medium used for cultivation, with cultures on TSA plates clearly being distinct from those on TSA + amoxicillin (Figure 3). Within each plate type, cultures were separated by the treatment group each nymph was raised in, with continuous transitions from nymphs raised in no amoxicillin through the MIC, 2xMIC, and 5xMIC for each plate type.

Taxon	0%	MIC	2xMIC	5xMIC
Acidobacteria	0.0025	0.0033	0.0025	0.0025
Actinobacteria	0.53	0.0033	0.0575	0
Armatimonadetes	0	0	0.0025	0
Bacteria_unclassified	0.0275	0.0033	0.115	0.0475
Bacteroidetes	0.265	0.03	0.34	0.03
Chloroflexi	0	0	0	0
Deinococcus-Thermus	0	0	0	0
Firmicutes	67.3925	22.5667	15.545	11.725
Planctomycetes	0	0	0.0525	0
Proteobacteria	31.78	77.4	83.885	88.1925

Table 3. Percentage of major bacterial phyla and subphyla associated with each sample raised on TSA plates. The averages of the four surviving nymphs in each of the four treatment groups (0% amoxicillin, MIC, 2xMIC, 5xMIC amoxicillin) are shown.

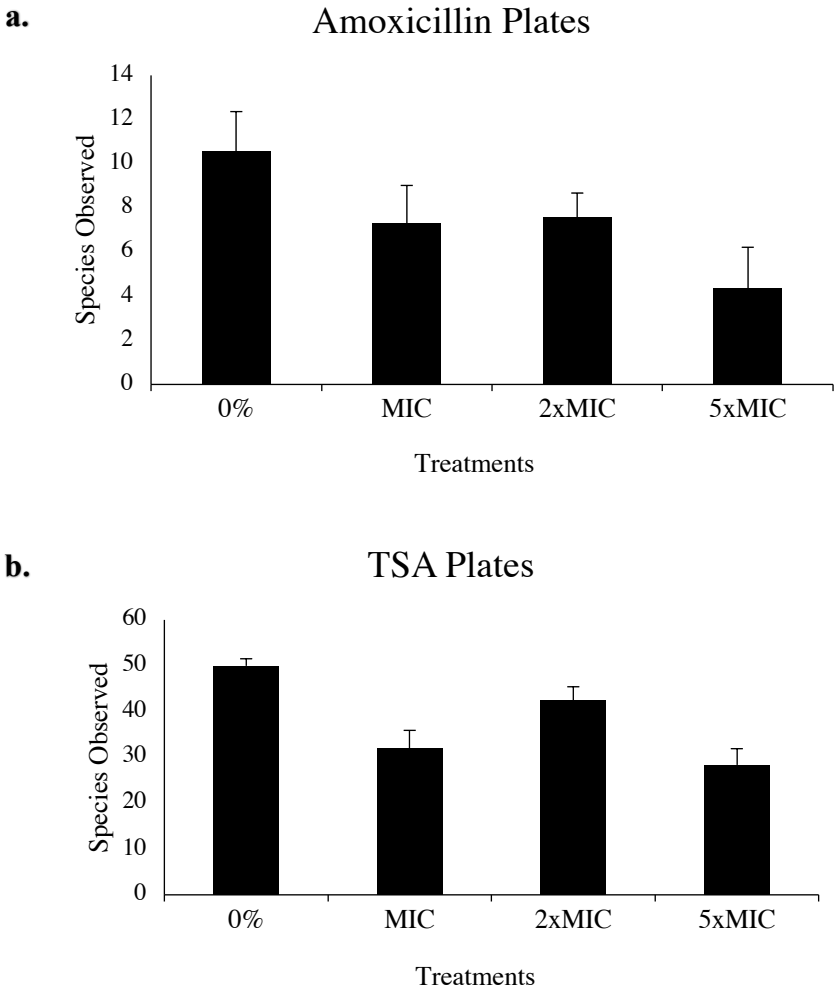

Figure 2. Diversity of cultures obtained from dragonfly nymphs raised in different levels of amoxicillin and assessed through observed species richness. Scores are calculated from next generation sequencing of pooled cultures on a plate. **Figure 2.a.** Mean number of species in cultures obtained from TSA + amoxicillin plates from nymphs raised in different concentrations of amoxicillin (0%, MIC, 2xMIC, and 5xMIC). **Figure 2.b.** Mean number of species in cultures obtained from TSA plates from nymphs raised in different concentrations of amoxicillin (0%, MIC, 2xMIC, and 5xMIC). The error bars represent the spread of the data within each treatment group around the mean value.

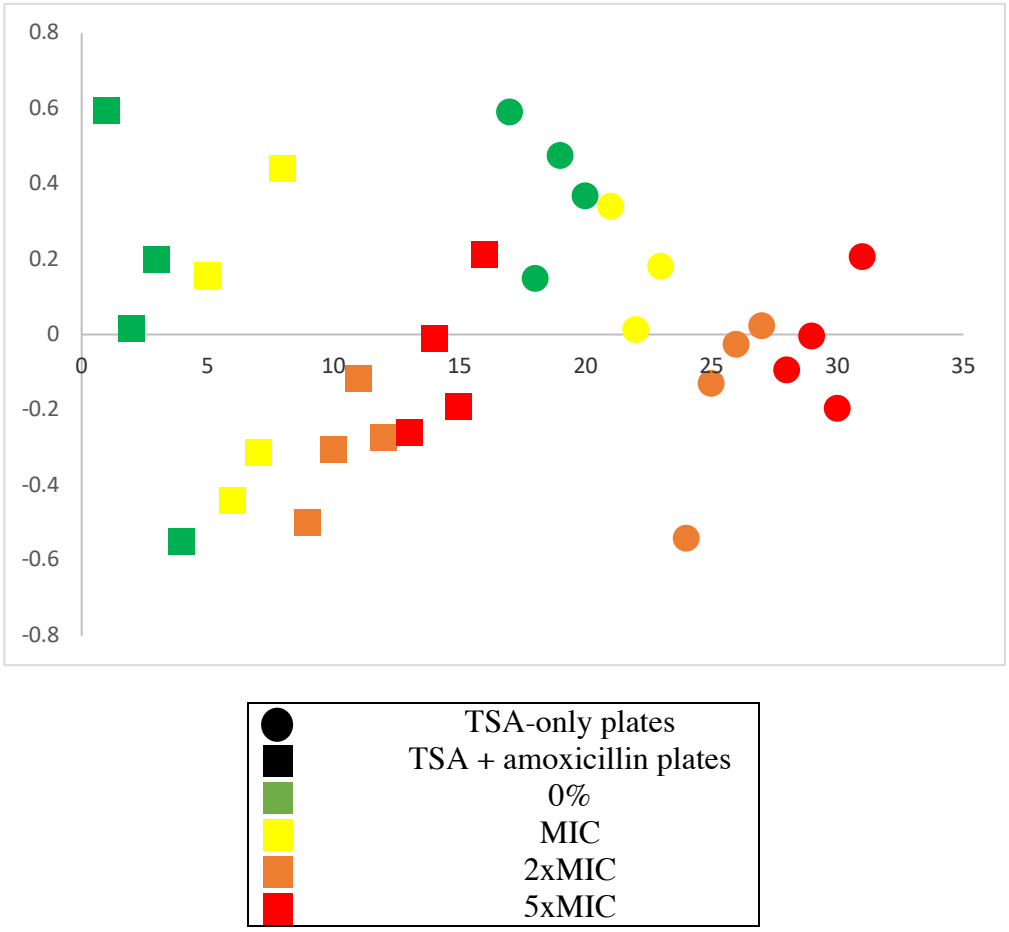

Figure 3. NMDS plot displaying the similarities between bacterial cultures obtained from dragonfly nymphs exposed to different levels of antibiotic and cultured on amoxicillin plates and TSA plates. Samples taken from nymphs raised in 0% amoxicillin are green; MIC yellow; 2xMIC orange; 5xMIC red. For each treatment, nymphs plated on TSA + amoxicillin plates are squares; circles represent samples plated on TSA-only plates.

Discussion

Bacterial communities associated with any living organism are affected by changes in the environment. The use of antibiotics in agriculture has proven to be a public health concern (Chang et al., 2015), even though the use of antibiotics in agriculture continues to rise (FDA, 2012). These antibiotics can enter agricultural runoff, which can enter local water systems (Wang et al., 2016), so that horizontal gene transfer of antibiotic resistance genes between environmental bacteria is possible (Davies, 1994). Dragonflies are a part of many aquatic food chains, so if exposure to antibiotics increases the presence of antibiotic resistant bacteria in these organisms, there is the possibility of conferring antibiotic resistant bacteria to other organisms that prey upon them such as fish (Uilenburg et al., 2006). This study investigated and compared the culturable bacterial communities associated with dragonfly nymphs exposed to different concentrations of the antibiotic amoxicillin.

Samples were taken from dragonfly nymphs raised in different concentrations of amoxicillin for ten days, in order to determine the bacterial communities of each of the samples. Based on the CFU counts it can be concluded that increased exposure to antibiotics results in more antibiotic resistant bacteria within dragonfly nymphs. This could be because specific bacteria became adapted and resistant to amoxicillin, or that existing bacterial species were replaced by those that showed amoxicillin resistance. Comparing the communities of cultured bacteria suggests the latter explanation, as the cultures clearly differed from nymphs raised on zero amoxicillin to those raised with increasingly higher concentrations of this antibiotic present. This was even apparent on

non-antibiotic plates, with the cultures of dragonfly nymphs differing in their bacterial communities between the different antibiotic treatment levels.

Nymphs were not identified to the species level, but were collected from the same habitat and selected based on similar size and appearance, with the assumption that they were the same species. To some extent, this assumption is supported by the culture identification, as the culturable bacterial communities collected from replicate nymphs in the same treatment were similar (or at least more similar than between treatments) (Tables 2 and 3). Host species has been shown to be a factor influencing microbiome composition, but as shown in this experiment, environmental conditions and the presence of potential contaminants is also important (Yun et al., 2014). The microbiome of insects can be important in resistance to pathogens, and acts not only to prevent infections, but to protect the insects from bacteria that may persist in their bodies (Haine et al., 2008). Thus, changes in the composition of the bacterial community within dragonfly nymphs, as induced by exposure to antibiotics, could have implications for the host organisms ability to respond to pathogens.

Proteobacteria was the dominant phylum of cultured bacteria, with the subphyla Betaproteobacteria and Gammaproteobacteria, making up the majority of all recovered cultures, respectively. Samples taken from both antibiotic and TSA plates also showed a high proportion of Firmicutes. This data is similar to that of previous studies on 218 different species of insects showing that insect gut microbiota are dominated by Proteobacteria (62.1%) and Firmicutes (20.7%) (Yun et al., 2014). Furthermore, Nair and Agashe (2016) cultured the gut microbiomes of dragonflies for various host species and found the order Enterobacteriales (phylum: Proteobacteria) to make up the majority of

their microbiomes. Members of the phylum Firmicutes (orders Bacillales and Lactobacillus) were also found to be in relatively high proportions (Nair and Agashe, 2016), similar to the results obtained here.

This study shows that increasing dragonfly nymphs' exposure to antibiotics leads to increased numbers of antibiotic resistant bacteria. It is possible that these bacteria can be retained into the dragonflies' adult life, although further studies would need to be conducted to support this hypothesis. If this is true, antibiotic resistant bacteria could be transmitted over long distances, as some adult dragonflies can migrate over hundreds to thousands of kilometers (May, 2013) and to different species of animals that prey on these adults. Antibiotic-resistant bacteria in the environment is an increasing public health concern, and aquatic ecosystems have been recognized as a reservoir for such bacteria. The number of antibiotic resistant bacteria can be increased by the trace amounts of antibiotics detected in waste water treatment plants, and water distribution systems may serve as an important reservoirs for the spread of antibiotic resistance (Xi, et al., 2009). This study suggests that organisms within aquatic ecosystems can be carriers of antibiotic resistant bacteria and this can be pronounced in environments subject to effluent containing antibiotics.

References

Andrews, JM (2001) Determination of minimum inhibitory concentrations. Journal of Antimicrobial Chemotherapy 48: 5-16.

Cabello, FC (2006) Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. Environmental Microbiology 8: 1137-1144.

CDC (2014) Outpatient antibiotic prescriptions. Centers for Disease Control 2.

Chang, Q, Wang, W, Regev-Yochay, G, Lipsitch, M, Hanage, WP (2015) Antibiotics in agriculture and the risk to human health: how worried should we be? Evolutionary Applications 8: 240-247.

Davies, J (1994) Inactivation of antibiotics and the dissemination of resistance genes. Science 264: 375-383.

Edelsberg, J, Weycker, D, Barron, R, Li, X, Wu, H, Oster, G, et al. (2014) Prevalence of antibiotic resistance in US hospitals. Diagnostic Microbiology and Infectious Disease 78: 255-262.

FDA (2012) Summary report on antimicrobials sold or distributed for use in food producing animals. Department of Health and Human Services 41.

Fleming-Dutra, KE, Hersh, AL, Shapiro, DJ, Bartoces, M, Enns, EA, File, TM, et al. (2016) Prevalence of inappropriate antibiotic prescriptions among US ambulatory care visits, 2010-2011. The Journal of the American Medical Association. 15: 1864-1873.

Fleming, A (1944) The discovery of penicillin. British Medical Bulletin 2: 4-5.

Glaser, L (2007) Dazzling dragonflies: A Life Cycle Story. Millbrook Press 7-9

Haine, ER, Moret, Y, Siva-Jothy, MT, & Rolff, J (2008) Antimicrobial defense and persistent infection in insects. Science 322: 1257-1259.

Khachatourians, GG (1998) Agricultural use of antibiotics and the evolution and transfer of antibiotic-resistant bacteria. Cmaj 159: 1129-1136.

Knight, TM, McCoy, MW, Chase, JM, McCoy, KA, Holt, RD (2005) Trophic cascades across ecosystems. Nature 437: 880-883.

Kozich, JJ, Westcott, SL, Baxter, NT, Highlander, SK, Schloss, PD (2013) Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the miseq illumina sequencing platform. Applied and Environmental Microbiology 79: 5112-5120.

Liu, P, Zhang, H, Feng, Y, Yang, F, Zhang, J (2014) Removal of trace antibiotics from wastewater: a systematic study of nanofiltration combined with ozone-based advanced oxidation processes. Chemical Engineering Journal 240: 211-220.

May, ML (2013) A critical overview of progress in studies of migration of dragonflies, with emphasis on North America. Journal of Insect Conservation 17: 1-15.

Nair, A, & Agashe, D (2016). Host-specific spatial and temporal variation in culturable gut bacterial communities of dragonflies. Current Science 8: 1513.

Pace, NR (1997) A molecular view of microbial diversity and the biosphere. Science 276: 734-740.

Schloss, PD, Westcott, SL, Ryabin, T, Hall, JR, Hartmann, M, Hollister, EB, et al. (2009) Introducing Mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and Environmental Microbiology 75: 7537-7541.

Spellberg, B, Guidos, R, Gilbert, D, Bradley, J, Boucher, HW, Scheld, WM (2008) The epidemic of antibiotic-resistant infections: a call to action for the medical community from the infectious diseases society of America. Clinical Infectious Diseases 46: 155-164.

Uilenberg, G (2006) Babesia—a historical overview. Veterinary Parasitology 138: 3-10.

Wang, J, Ben, W, Yang, M, Zhang, Y, Qiang, Z (2016) Dissemination of veterinary antibiotics and corresponding resistance genes from a concentrated swine feedlot along the waste treatment paths. Environment International 92: 317-323.

Waldor, MK, Tyson, G, Borenstein, E, Ochman, H, Moeller, A, Finlay, BB, et al. (2015) Where next for microbiome research? PLoS Biology 13: 4-15. doi.org/10.1371/journal.pbio.1002050.

Wang, Y, Gilbreath III, TM, Kukutla, P, Yan, G, Xu, J (2011) Dynamic gut microbiome across life history of the malaria mosquito *Anopheles gambiae* in Kenya. PLoS One 6. doi.org/10.1371/journal.pone.0024767

Woese, CR (1987) Bacterial evolution. Microbiological Reviews 51: 221-225.

Xi, C, Zhang, Y, Marrs, CF, Ye, W, Simon, C, Foxman, B, et al. (2009) Prevalence of antibiotic resistance in drinking water treatment and distribution systems. Applied and Environmental Microbiology *75*: 5714-5718.

Yun, JH, Roh, SW, Whon, TW, Jung, MJ, Kim, MS, Park, DS, et al. (2014) Insect gut bacterial diversity determined by environmental habitat, diet, developmental stage, and phylogeny of host. Applied and Environmental Microbiology 80: 5254-5259.